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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	SMITH et al.
Serial No.	:	09/484,886
Filed	:	January 18, 2000
Title	:	APPARATUS AND METHODS OF PRODUCING AND USING HIGH DENSITY CELLS AND PRODUCTS THEREFROM
Group Art Unit	:	1651
Examiner	:	Kailash C. Srivastava

DECLARATION OF MANON COX

Commissioner for Patents
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Alexandria. VA 22313-1450

Entry recommended 3/17/2005

Dear Sir:

MANON COX DECLARES AND SAYS THAT:

I am qualified to speak as to the instant invention and the data presented

1. My *Curriculum vitae* is attached as Exhibit A. I respectfully submit that I am skilled in the art to which the present application pertains. I am advised that claims currently pending in this application are directed to a substantially pure, recombinant glycosylated erythropoietin produced by a baculovirus expression system in cultured insect cells, wherein said erythropoietin has relative homogeneity or is purified to 95% or greater and said erythropoietin stimulates erythropoiesis and has an activity of at least 200,000 U/mg or of about 500,000 U/mg. I further understand that the previously pending claims were rejected as anticipated and obvious over Quelle et al. with evidence provided by Dorland's Illustrated Medical Dictionary. I further understand that the claims now pending are directed to a substantially pure, recombinant glycosylated erythropoietin produced by a baculovirus expression system in cultured insect cells, wherein said erythropoietin has relative homogeneity or is purified to 95% or greater and said erythropoietin stimulates erythropoiesis and has an activity *in vivo* of at least 200,000 U/mg or of about 500,000 U/mg, as set forth, or as substantially set forth, in Exhibit B. In addition, work reported herein was performed under my direction, supervision or control in the ordinary course of business. I respectfully submit that I am thus qualified to introduce the work reported herein.

I further respectfully submit that in view of my education, training and experience, as well as my understanding as to the now pending claims, and my ability to speak as to the work reported herein, that I am qualified to render opinions as to the state of the art to which the present invention pertains and as to the present invention.

2. More specifically, this Declaration is submitted in support of the patentability of the now pending claims; and, I respectfully submit that the now pending claims are patentable as the presently claimed invention is not anticipated and is nonobvious and surprising because one skilled in the art could not predict that the erythropoietin of the present invention would be active *in vivo* and would stimulate erythropoiesis *in vivo*, for instance, as shown by the data below, wherein the erythropoietin of the cited references does not have activity *in vivo* and does not stimulate erythropoiesis *in vivo*.

The superior utility of the instant invention

3. Human erythropoietin, a glycoprotein with a native molecular weight of 36,000 daltons stimulates erythropoiesis, or red blood cell production. Produced in the kidney, it targets progenitor erythroid cells in bone marrow to divide and differentiate into red blood cells. Normal circulating levels of EPO range between 0.01-.03 units/ml plasma; reaching up to 1000 fold higher levels during peak physiological demand.

Its current clinical use is to overcome anemia associated with chronic renal failure and cancer chemotherapy. The licensed product is manufactured in CHO cells engineered to synthesize EPO.

The insect-cell derived EPO of the present invention, with a 165 amino acid core identical to the human protein and a M_r of 26,000 daltons, such as that produced in Protein Science Corporation's BEVS, exists as a protein with a glycosylation pattern differing from the native human form. Insect-cell derived EPO lacks the highly branched sugar side chains terminating in sialic acid that characterizes the human protein. Instead, the sugar side chains are unbranched with no terminal sialic acid. Articles such as Quelle have characterized the "lack of in vivo activity" of insect-cell derived EPO as a function of the absence of sialic acid. Quelle at 656. However, investigations into PSC BEVS EPO bio-activity parallel those observations of similar natural EPO molecules reported in the literature. The insect-cell derived EPO of the present invention has exceptionally high *in vitro* activity, and is active in the routine *in vivo* bioassays used to determine the specific activity of EPO.

Studies of EPO activity

4. Studies of BEVS produced EPO activity have been performed utilizing several in vitro and in vivo tests.

a. *In vitro* bio-assays: Cell proliferation and 3H-thymidine uptake

BEVS EPO activity was determined in two *in vitro* assay systems, each utilizing a different cell line dependent on EPO during cell growth. One assay measured cell proliferation and the other measured 3H-thymidine uptake. The average activity for BEVS EPO exceeded 574,000U/mg, while a representative commercial CHO-EPO activity was 126,000U/mg

b. *In vivo* bio-assays: Single mice were administered a single dose of test EPO, then 96 hours later, blood samples were extracted and reticulocytes levels were determined by flow cell-cytometry, as a direct measure of erythropoiesis.

In a side-by-side comparison with CHO manufactured EPO, PSC EPO lacked activity.

Table 1[1] Single dose on day one only

Treatment	N	Dose Frequency (doses per day)	# days dosed	Route	Dose /day ng/mouse	µg/kg	Response (reticulocytes / 2 X 10^6 counts)
Epogen	8	1	1	SC	80	2.9	5596
	8	1	1	SC	160	5.8	7617
	8	1	1	SC	320	11.6	9191
PSC EPO	8	1	1	SC	50	2	3608
	8	1	1	SC	100	4	3769
	8	1	1	SC	200	6	3648
PBS							3435

1. average weight: 25-30g
2. dose volume was constant at 0.5ml
3. historical control value

c. *In vivo* bio-assays: Single Dose

Mice were administered a single dose of test EPO with higher doses of PSC EPO than used in the above Experiment, then 96 hours later, blood sample were extracted and reticulocytes levels were determined as a direct measure of erythropoiesis.

In a side-by-side comparison with CHO manufactured EPO, PSC EPO lacked activity.

Table 2 Single dose on day one only

Treatment	N	Dose Frequency (doses per day)	# days dosed	Route	Dose /day ng/mouse	µg/kg	Response (reticulocytes / 2 X 10^6 counts)
Epogen	3	1	1	SC	64	3.2	8,364
	3	1	1	SC	128	6.4	12,838
	3	1	1	SC	256	12.8	15,216
PSC EPO	3	1	1	SC	160	8	5,148
	3	1	1	SC	500	25	5,182
	3	1	1	SC	1,600	80	4,700
PBS control	4	1	1				5,356

1. average weight 20g
2. dose volume was constant at 0.5ml

d. *In vivo* bio-assays: Range Finding bio-assay: Repeated Once-Daily Dosing

Mice were administered one dose daily for four days, then 96 hours after the first dose, blood sample were extracted and reticulocytes levels were determined as a direct measure of erythropoiesis. In a direct comparison with CHO manufactured EPO, PSC-EPO demonstrated reduced activity, with only the highest dose group, 2500 µg/kg demonstrating a significant difference between the control group and the next lowest treatment group.

Table 3. Single doses repeated once each day for four days.

Treatment	N	Dose Frequency (doses per day)	# days dosed	Route	Dose /day ng/mouse	µg/kg	Response (reticulocytes / 2 X 10^6 counts)
Epogen	3	1	4	IP	75	3.75	37,134
	3	1	4	IP	150	7.5	41,400
PSC EPO	3	1	4	IP	500	25	8534
	3	1	4	IP	2,000	100	8934
	3	1	4	IP	10,000	500	10,266
	3	1	4	IP	50,000	2500	14,134
PBS	9	1	4	IP			8,266

1. average weight 20g
2. dose volume was constant at 0.5ml

Figure 1 depicts the dose response to Epogen (<), PSC BEVS EPO (*) and PBS (....) after once daily dosing for four days, expressed as reticulocytes counts as a percentage of 200,000 total counts. Samples were collected for analysis 96 hours after the first dose. The dashed line represents the average response to the saline control.

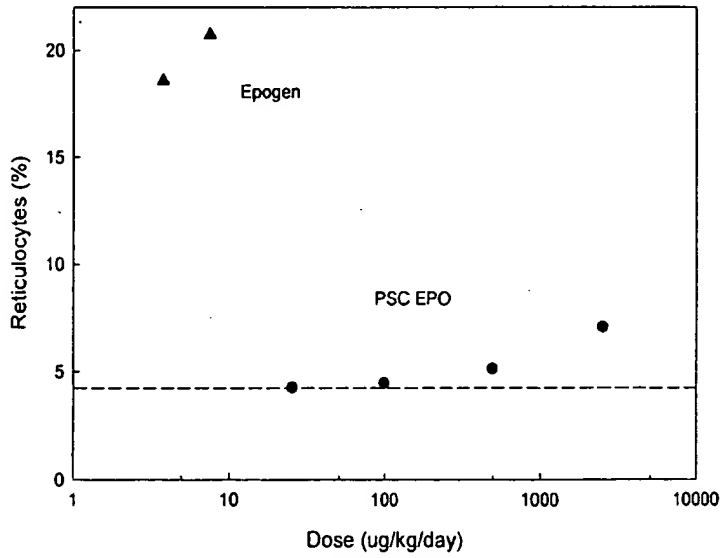

FIGURE 1

e. Range Finding bio-assay: Repeated Multiple doses each day

Mice were administered three doses daily at eight hour intervals for four days, then 96 hours after the first dose, blood sample were extracted and reticulocytes levels were determined as a direct measure of erythropoiesis.

PSC EPO demonstrated a dose response relationship over the dose range tested. For PSC EPO, maintaining the identical total daily dose level, but dividing into three equal doses applied at 8 hour intervals, increased the response almost two-fold. For Epogen, a similar dose schedule had no effect on overall response. The dose of Epogen required to produce a response similar to PSC EPO was approximately 1,000 times smaller than the PSC EPO dose.

Table 4. Three doses each day for 4 days as a means of maintaining effective circulating EPO levels.

Treatment	N	Dose Frequency (doses per day)	# of days	Route	Dose - each injection (µg/kg)	Daily Dose (µg/kg/day)	Dose - each injection (ng/mouse)	Daily Dose (ng/m/day)	Response (reticulocytes / 2 X 10^5 counts)
Epogen	3	3	4	I.P.	0.75	2.25	15	45	31,600
	3	1	4	I.P.	2.25	2.25	45	45	31,600
PSC EPO	3	3	4	I.P.	200	600	4,000	12,000	15,000
	3	3	4	I.P.	450	1,350	9,000	27,000	19,400
	3	3	4	I.P.	1,000	3,000	20,000	60,000	23,800
	3	1	4	I.P.	3,000	3,000	60,000	60,000	13,600
PBS	7	3	4	I.P.					10,200

1. 8 hour dose intervals, 8:00am, 4:00pm and 12:00am
2. Average mouse weight: 22
3. Dose volume: 0.25ml

Figure 2 depicts the dose response to PSC BEVS EPO ( )) and PBS (----)after three doses daily dosing for four days, expressed as reticulocytes counts as a percentage of 200,000 total counts. Samples were collected for analysis 96 hours after the first dose. (•) represents the

response to a single dose of PSC EPO repeated for four days. ($\prod$) represents the response to CHO EPO both one and three times per day. The dashed line represents the average response to the saline control.

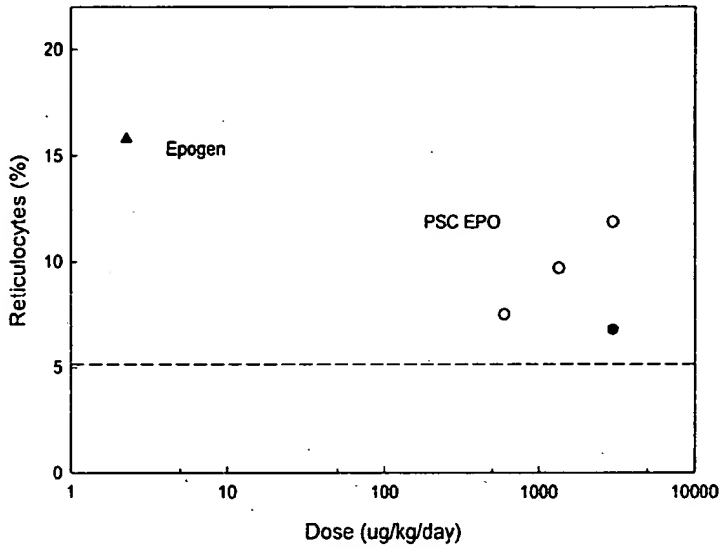

FIGURE 2

f. Two different routes of administration have been used in these experiments, subcutaneous (sc) and intra-peritoneal (ip). In both cases, absorption is dependent on uptake by blood and lymphatic capilaries; small molecules are absorbed directly into blood capilaries, proteins like EPO are taken into lymphatic channels. There possibly are slight differences in rates of absorption from these different sites. Absorption of drugs after subcutaneous administration depends on the vascularity of the site; total surface area over which absorption can occur is the prime determinant of the rate of absorption. Also, lateral flow is limited by tissue matrix. Factors that increase or decrease blood flow also affect the rate of absorption. Absorption from the peritoneal cavity is dependent on co-mixing with other peritoneal fluids followed by diffusion across peritoneal membranes. Overall, variability associated with dosing is less when using the ip route.

g. The dose volume was reduced from 0.5ml to 0.25ml in an attempt to reduce possible volume related stress associated with multiple injections.

h. As shown above, use of the insect-cell derived EPO of the present inveniton results in increased reticulocyte counts. As described previously, reticulocyte counts are deemed a direct measure of erythropoiesis. The clear observation is that the more frequest the dose, the greater the resposne, the greater the total of whole blood reticulocytes, and thereby the greater *in vivo* stimulation of erythropoiesis.

The present invention is therefore not obvious

5. It is therefore respectfully submitted that the inventive substantially pure, recombinant glycosylated insect-cell derived EPO is not obvious as one could not have predicted that the inventive EPO would would have achieved the results that it does, e.g., increased reticulocyte counts, indicating *in vivo* acticity and stimulation of erythropoiesis, e.g., as shown by the results above.

The present invention is therefore not anticipated

6. It is also therefore respectfully submitted that the inventive substantially pure, recombinant glycosylated insect-cell derived EPO is not anticipated because as shown above, the EPO of the present invention results in increased reticulocyte counts, indicating *in vivo* activity and stimulation of erythropoiesis regardless of the lack of sialic acid which Quelle concludes in necessary for *in vivo* activity.

7. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

Dated: March 18, 2004 By: Manon Cox
MANON COX

APPENDIX A

Personal Details

Name: Manon M.J. Cox
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Education and Qualifications

Date	College	Qualifications
1976 -1981	St. Maarten College	
1981-1987	University of Nijmegen The Netherlands	"Doctorandus" Degree in Biology Specialization: Molecular Biology, Genetics and Biochemistry
1990-1993	Open University	Economics (various subjects)
1996-1997	University of Nijenrode & University of Rochester (NY)	Master of Business Administration

Current Position — **Protein Sciences Corporation**

July 2002 - present — Chief Operating Officer

- Responsible for all operations of company, including research and development, manufacturing and QA/QC.

June 1998- July 2002 — Vice President, Corporate and Process Development.

- Manage and direct all research and process development activities.
- Develop and create policies and programs within the U.S., Europe and Asia in the field of complex recombinant proteins.
- Identify and manage joint business projects with third parties.
- Responsible for supervising the Research and Business Development operations.

Career Details — **Gist-Brocades N.A**

July 1995-June, 1998 — Manager New Business Development *Biopharmaceuticals.*

- Prepared business plan for Gist-brocades' entrance into this market as the manufacturing partner of choice.
- Manage and direct Research and Development, production, market research and supporting staff (e.g. patents and trademarks) to realize goals set in the business plan.
- Explore, identify and evaluate potential new projects within the scope of the business plan.
- Identify and manage joint business projects with third parties (from the pharmaceutical industry).
- License Gist-brocades' expression technology to third parties.
- Budget responsibility -- 1997: 3 mil. Dutch guilders.

July 1994 - July 1995 Manager Department Process Development (Bruges)
The group (consisting of 13 people) is responsible for the optimization of production processes, the implementation of new processes and trouble shooting in production.

Sept.1993 - June 1994 Management Trainee
Responsible for the implementation of the concept "horizontal management" in multidisciplinary project teams, where the interaction between the different disciplines is crucial for success (e.g. logistics: co-operation between production and sales forecasting).

Sept.1991 - Sept.1993 Project Manager Research & Development
Responsible for the development of a detergent enzyme.

The project team consisted of 8 researchers and the project manager was part of a larger multidisciplinary team (e.g. Research and Development. Production, Engineering and Marketing and Sales)

Sept.1988 - Aug.1991 Scientist, Dept. Bacterial Genetics (Gist-brocades)

Sept.1987- Aug.1988 Scientist University of Amsterdam

A list of publications (including a patent) can be made available upon request

APPENDIX B

96. (Currently Amended) A substantially pure, recombinant glycosylated erythropoietin, produced by a baculovirus expression system in cultured insect cells, wherein said erythropoietin has relative homogeneity or is purified to 95% or greater and said erythropoietin stimulates erythropoiesis and has an activity *in vivo* of at least 200,000 U/mg or of about 500,000 U/mg.

97. (Currently Amended) Erythropoietin of claim 96 wherein said erythropoietin stimulates erythropoiesis and has an activity *in vivo* of at least 200,000 U/mg.

98. (Currently Amended) Erythropoietin of claim 96 wherein said erythropoietin stimulates erythropoiesis and has an activity *in vivo* of at least 500,000 U/mg.

99. (Previously added) Erythropoietin of claim 96 produced by a method comprising:

culturing insect cells in at least one bioreactor whereby there is an insect cell culture,

wherein the insect cells contain a recombinant baculovirus containing exogenous DNA encoding erythropoietin,

supplying medium in at least one vessel whereby there is culture medium,

circulating culture medium and/or insect cell culture, whereby the bioreactor and vessel are in fluid communication and the insect cell culture and/or culture medium are in circulation,

delivering oxygen to the insect cell culture and/or culture medium, and

collecting the expressed product, and/or baculovirus and/or the cells.

100. (Previously added) Erythropoietin of claim 96 produced by a method comprising:

culturing insect cells in a bioreactor whereby there is an insect cell culture,

wherein the insect cells contain a recombinant baculovirus containing exogenous DNA encoding erythropoietin,

supplying culture medium in a vessel whereby there is culture medium,

circulating the insect cell culture through a dialysis means,

circulating culture medium through the dialysis means,

wherein the dialysis means in fluid communication with the bioreactor and the vessel,

whereby

there is

a first, cell culture, loop between the bioreactor and the dialysis means, and

a second, media replenishment, loop between the vessel and the bioreactor,

performing dialysis between the culture medium and the cell culture, and

collecting the erythropoietin.

101. (Previously added) Erythropoietin as claimed in claim 100, wherein the method further comprises:

delivering oxygen into the cell culture loop and measuring physical and/or chemical parameter(s) of the cell culture and/or the culture medium.

102. (Previously added) Erythropoietin as claimed in claim 101, wherein the method further comprises adjusting physical and/or chemical parameter(s) of the cell culture and/or the culture medium in response to data from the measuring.

103. (Previously added) Erythropoietin as claimed in claim 101, wherein the method further comprises measuring pH and measuring dissolved oxygen concentration, adjusting physical and/or chemical parameter(s) of the cell culture and/or the culture medium in response to data from the measuring, wherein the adjusting comprises adjusting temperature to maintain a desired temperature, adjusting pH to maintain a desired pH, and adjusting dissolved oxygen concentration and dissolved carbon dioxide concentrations, whereby the dissolved carbon dioxide levels are adjusted in response to pH measurement(s).

104. (Previously added) Erythropoietin as claimed in claim 103, wherein the method further comprises adjusting dissolved oxygen levels in response to dissolved oxygen measurement(s), adjusting pH to a desired level in response to pH measurement(s) by adjusting the dissolved carbon dioxide concentration such that dissolved carbon dioxide concentration is adjusted when pH varies from the desired level, and the dissolved oxygen measurement varies periodically as a function of time, adjusting the dissolved oxygen concentration so that the dissolved oxygen measurement varies from 30% to 90% or from 40% to 80% or from 50% to 70%; or, so that the dissolved oxygen measurement averages about 60%.

105 (Previously added) Erythropoietin as claimed in claim 104, wherein the adjusting of the dissolved oxygen concentration so that the dissolved oxygen measurement varies from 30% to 90%.

106 (Previously added) Erythropoietin as claimed in claim 104, wherein the adjusting of the dissolved oxygen concentration so that the dissolved oxygen measurement varies from 40% to 80%.

107 (Previously added) Erythropoietin as claimed in claim 104, wherein the adjusting of the dissolved oxygen concentration so that the dissolved oxygen measurement varies from 50% to 70%.

108 (Previously added) Erythropoietin as claimed in claim 104, wherein the adjusting of the dissolved oxygen concentration so that the dissolved oxygen measurement averages about 60%.

109. (Previously added) Erythropoietin as claimed in claim 104, wherein the method further comprises adjusting the dissolved oxygen concentration so that the dissolved oxygen measurement varies from high value to low value over about 10 to about 30 minutes or over about 20 minutes.

110. (Previously added) Erythropoietin as claimed in claim 103, wherein the method further comprises adjusting dissolved oxygen levels in response to dissolved oxygen measurement(s), and adjusting pH to a desired level in response to pH measurement(s) by adjusting the dissolved carbon dioxide concentration such that dissolved carbon dioxide concentration is adjusted when pH varies from the desired level, and the dissolved oxygen measurement varies periodically as a function of time, and wherein a plot of the dissolved oxygen measurement as a function of time comprises a sine wave.

111. (Previously added) Erythropoietin as claimed in claim 99 wherein the insect cells are *Spodoptera frugiperda* cells.

112. (Previously added) Erythropoietin as claimed in claim 100 wherein the insect cells are *Spodoptera frugiperda* cells.

113. (Previously added) Erythropoietin as claimed in claim 111 wherein the medium is serum free.

114. (Previously added) Erythropoietin as claimed in claim 112 wherein the medium is serum free.

115. (Previously added) Erythropoietin as claimed in claim 111 wherein the insect cells are *Spodoptera frugiperda* SF900+ cells.

116. (Previously added) Erythropoietin as claimed in claim 112 wherein the insect cells are *Spodoptera frugiperda* SF900+ cells.

117. (Withdrawn) An expressed product obtained by a method for growing cells comprising

culturing cells in at least one bioreactor whereby there is a cell culture,

supplying medium in at least one vessel whereby there is culture medium,

circulating culture medium and/or cell culture, whereby the bioreactor and vessel are in fluid communication and the cell culture and/or culture medium are in circulation, and

delivering oxygen to the cell culture and/or culture medium,

wherein the cells contain a vector for replication of the vector and/or expression of exogenous nucleic acid molecules wherein the vector comprises a virus or a recombinant virus;

wherein the method further comprises collecting expressed product, and/or baculovirus and/or the cells.

118. (Withdrawn) An expressed product obtained by a method for growing cells comprising

culturing cells in a bioreactor whereby there is a cell culture, wherein the cells contain a vector for replication of the vector and/or expression of exogenous nucleic acid molecules wherein the vector comprises a virus or a recombinant virus,

supplying culture medium in a vessel where by there is culture medium,

circulating the cell culture through a dialysis means,

circulating culture medium through the dialysis means,

wherein the dialysis means in fluid communication with the bioreactor and the vessel,

whereby

there is

a first, cell culture, loop between the bioreactor and the dialysis means, and

a second, media replenishment, loop between the vessel and the bioreactor,

performing dialysis between the culture medium and the cell culture,

and collecting expressed product, and/or baculovirus and/or the cells.

119. (Withdrawn) The expressed product of claim 118, wherein the method by which the product is obtained further comprises

delivering oxygen into the cell culture loop,

and measuring physical and/or chemical parameter(s) of the cell culture and/or the culture medium.

120. (Withdrawn). The expressed product of claim 119, wherein the method by which the product is obtained further comprises

adjusting physical and/or chemical parameter(s) of the cell culture and/or the culture medium in response to data from the measuring.

121. (Withdrawn) The expressed product of claim 120, wherein the adjusting comprises adjusting temperature to maintain a desired temperature, and wherein the adjusting comprises adjusting dissolved oxygen concentration and adjusting dissolved carbon dioxide concentration, whereby in response to pH measurement(s), dissolved carbon dioxide levels are adjusted.

122. (Withdrawn) The expressed product of claim 121 wherein the adjusting includes adjusting dissolved oxygen levels in response to dissolved oxygen measurement(s), wherein the adjusting comprises adjusting pH to a desired level in response to pH measurement(s) by adjusting the dissolved carbon dioxide concentration such that dissolved carbon dioxide concentration is adjusted when pH varies from the desired level, and the dissolved oxygen measurement varies periodically as a function of time, and wherein the adjusting includes adjusting the dissolved oxygen concentration so that the dissolved oxygen measurement varies from 30% to 90% or from 40% to 80% or from 50% to 70%; or, so that the dissolved oxygen measurement averages about 60%.

123. (Withdrawn) The expressed product of claim 122, wherein the adjusting includes adjusting the dissolved oxygen concentration so that the dissolved oxygen measurement varies from high value to low value over about 10 to about 30 minutes or over about 20 minutes.

124. (Withdrawn) The expressed product of claim121, wherein the adjusting includes adjusting dissolved oxygen levels in response to dissolved oxygen measurement(s), wherein the adjusting comprises adjusting pH to a desired level in response to pH measurement(s) by adjusting the dissolved carbon dioxide concentration such that dissolved carbon dioxide concentration is adjusted when pH varies from the desired level, and the dissolved oxygen